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(57) Abstract

Embryonic stem (ES) cells are isolated from a cultured embryo containing both ES cells and differentiated cells by selectively killing the differentiated cells *in situ*. This improves the efficiency of the existing (manual) technique, allowing derivation of ES cell lines in species which have to date proved intractable. The killing may be achieved either by a drug selectable marker system or by expression of a lethal gene. In the former case, undiferentiated ES cells are protected against the toxic effects of a drug by selective expression of a drug resistance marker (such as *neo*). In the latter case, the lethal gene (such as the diphtheria toxin gene) is selectively expressed in differentiated cells. Expression cassettes may subsequently be removed by site-specific recombination systems.

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EMBRYONIC STEM CELL ISOLATION

This invention relates to a method for isolating embryonic stem cells.

Mouse embryonic stem (ES) cells are rapidly proliferating, undifferentiated, totipotent cells derived from early embryos. They can be grown in large numbers *in vitro* and then reintroduced into an early embryo where they can contribute to both somatic and germ cells of a chimeric animal. ES cells are becoming widely used as a route by which genetic alterations wrought *in vitro* can be introduced into mice and then stably inherited. A particularly powerful approach is the generation of predetermined genetic modifications by gene targeting in ES cells. This makes it possible to inactivate, alter, or replace a gene of interest (Bradley *et al.*, *Bio/Technology* **10** 534-539 (1992)). Applications include: fine analysis of regulation of gene expression, analysis of protein structure and function, generation of animal models of human diseases and precise placement of heterologous genes in transgenic animals.

Derivation of an ES cell line from a murine embryo is essentially the release of a normally transient population of undifferentiated cells from the controlling influence of the embryonic environment. ES cells have the innate capacity to proliferate indefinitely without any form of oncogenic transformation.

The standard method of obtaining a murine ES cell line was devised by Evans *et al.* (*Nature* **292** 154-156 (1981)) and independently by Martin (*Proc. Nat'l. Acad. Sci. USA.* **78** 7634-7638 (1981)) and is described in detail by

Robertson, E.J. "Embryo-derived stem cells" in "Teratocarcinomas and Embryonic Stem Cells, A Practical Approach". Ed. E.J. Robertson. Pub. IRL Press Ltd, Oxford, 1987. Briefly, blastocyst stage embryos are flushed from the uterus and then cultured in medium over a layer of mitotically inactivated fibroblasts, "feeder" cells. Over a few days blastocysts attach and spread onto the feeder layer, exposing cells of the inner cell mass. The inner cell mass of some explants proliferate forming clumps of undifferentiated cells. This portion of each explant is manually isolated, disaggregated and replated onto a new feeder layer. Colonies of cells form, usually composed of several cell types. Any colonies with ES morphology are further isolated, disaggregated and replated. Repeating this through several passages can result in a homogeneous population of ES cells.

A vital part of the derivation process is the physical separation of ES cells away from differentiated tissues, e.g. trophectoderm and endoderm. These arise from differentiated cells of the explanted embryo and also from spontaneous differentiation of ES cells. Endoderm is commonly the first product of ES cell differentiation and cells of this type tend to lie closely apposed to undifferentiated ES cells. The proximity of differentiated cells induces further differentiation of ES cells.

The presence of feeder cells is necessary during the first stages of ES cell derivation to reduce spontaneous differentiation of undifferentiated cells. However, the mechanism involved is only partially defined. Established ES cell lines can usually be grown

successfully in the absence of a feeder layer (Magin *et al.*, *Nuc. Acids. Res.* **14** 3795 (1992)), providing that the culture medium is supplemented with the cytokine leukaemia inhibitory factor, LIF (Smith *et al.*, *Nature* **336** 688-689 (1988) and Williams *et al. Nature* **336** 684-687 (1988)). However soluble LIF alone is not usually a substitute for feeders during ES cell derivation.

The frequency with which ES lines are derived from embryos varies widely. In skilled hands ES lines can be obtained from 5-10% mouse blastocysts using strain 129Sv (Robertson, E.J., M.H. Kaufman., Bradley, A and M.J. Evans. 1983. "Isolation, Properties and Karyotype Analysis of Pluripotential (EK) Cells from Normal and Parthenogenetic Embryos" in: "Teratocarcinoma Stem Cells", *Cold Spring Harbor Conferences on Cell Proliferation* **10** 647-663. Eds. Martin, G.R., L. Silver and S. Strickland.

It would be highly desirable to extend the use of gene targeting in ES cells to animals with greater commercial utility than mice, particularly rats and domestic animals. Several researchers have attempted to generate ES cells from farm animals (Handyside *et al.*, *Roux's Arch. Dev. Biol* **196** 185-190 (1987), Notorianni *et al.*, *J. Reprod. Fertil. (Suppl.)* **41** 51-6 (1990), Piedrahita *et al.*, *Theriogenology* **34** 865-877 (1990), Piedrahita *et al Theriogenology*. **34** 879-901 (1990) and Salto *et al.*, *Roux's Arch. Dev. Biol.* **201** 134-141 (1992)), but success has been limited. While ES-like cells have been derived, there are no reports that any are capable of contributing to a developing embryo to form a chimeric animal.

A common difficulty in the derivation and maintenance of ruminant ES cells is the progressive differentiation of undifferentiated cells which leads to eventual extinction of the cell line. It is now proposed that the differentiation of ES cells is in large part induced by adjacent differentiated cells. Hitherto, as explained above, differentiated cells have, where possible, been manually separated from ES cells. The present invention is based on the realisation that the ablation of differentiated cells, that is to say the specific destruction of differentiated cells *in situ*, increases the efficiency with which ES cells may be derived from embryos.

According to a first aspect of the present invention there is provided a method for selecting embryonic stem (ES) cells from an embryo in culture, the method comprising selectively killing differentiated cells of the embryo.

The differentiated cells may be derived from the embryo as explanted into the (generally *in vitro*) culture or may result from the differentiation of ES cells within the embryo. The intention will generally be to selectively kill all differentiated cells.

The invention has application to the production not only of murine stem cells but also stem cells of other animals, particularly other placental mammals, such as rats (*Ratus* spp.), other rodents, rabbits and domesticated animals, particularly farm animals such as cattle, pigs, sheep and goats. The invention is not, however, limited to any particular species and may be found to have a very wide range of applicability across the animal kingdom.

Explanted embryos will generally contain undifferentiated (ES) cells as well as differentiated cells such as trophectoderm and endoderm cells. The explanted embryo may be obtained, as outlined by Robertson (1987) *loc. cit.*, by abstracting an embryo from the uterus (for example at the blastocyst stage) and culturing in a suitable medium over a layer of feeder cells, which may be mitotically inactivated fibroblasts, as previously described. The explanted embryo on which the selective killing step of the invention is performed may have gone through one or more passages in which a clump of predominantly undifferentiated cells is isolated, disaggregated and replated onto a new feeder layer.

In one preferred embodiment of the invention, the differentiated cells are killed by means of a drug selection regime. The regime may be used to kill differentiated cells during early passages of the explanted embryo. Undifferentiated ES cells may express a resistance marker for a drug which is toxic to both differentiated and undifferentiated cells, so that when the colony is exposed to the drug only the differentiated cells are killed. For this embodiment, the use of a promoter specific or substantially specific for undifferentiated cells is important, and the embryo cells can be made transgenic for, or otherwise be able to express, a construct comprising such a promoter operatively coupled to a DNA sequence encoding the drug resistance marker.

There have been two published reports of genes which are strongly down-regulated on the differentiation of ES cells. These are the transcription factor Oct-3/4 (Okazawa *et al.*, *EMBO J.* **10** 2997-3005 (1991)) and the

growth factor FGF-4 (Ma *et al Dev. Biol.* **154** 45-54 (1992)). The examples which appear below use the Oct-3/4 promoter, although the FGF-4 promoter could also be used, as could any other suitable ES-specific promoters.

The transcription factor Oct-3/4 was first identified as present in undifferentiated mouse embryonal carcinoma (EC) cells (closely related to ES cells) but not in their differentiated derivatives (Okamoto *et al.*, *Cell* **60** 461-472 (1990)). Analysis of Oct-3/4 mRNA expression in mouse showed that it was restricted to undifferentiated ES and EC cells (Ben-Shusan *et al.*, *Mol. Cell. Biol.* **13** 891-901 (1993)), embryo inner cell mass and primordial germ cells (Rosner *et al.*, *Nature* **345** 686-92 (1990)). The Oct-3/4 promoter is capable of conferring Oct-3/4 specific expression on a heterologous reporter gene in transgenic mouse embryos (Okazawa *et al.*, *EMBO J.* **10** 2997-3005 (1991)).

Several genes are available for conferring drug resistance on non mutant cells, e.g. G418 selection for the *neo* gene (Colbere-Garapin *et al.*, *J. Mol. Biol.* **150** 1-14 (1981)), hygromycin selection for the *hygro* gene (Santerre *et al.*,*Gene* **30** 147-156 (1984)), histidinol selection for the *his* gene (Hartman *et al.*, *Proc. Nat'l. Acad. Sci.* **85** 8047-8051 (1988)), methotrexate selection for the *dhfr* gene (Wigler *et al.*, *Proc. Nat'l. Acad. Sci. USA* **77** 3567-3570 (1980)), aminopterin/mycophenolic acid selection for the *gpt* gene (Mulligan *et al.*, *Proc. Nat'l. Acad. Sci. USA* **78** 2072-2076 (1981)), methionine sulphoximine selection for the glutamine synthetase (*gs*) gene (Hayward *et al.*, *Nucl. Acids Res.* **14** 999-1008 (1986)) and deoxycoformicin selection for the adenosine deaminase (*ada*) gene (Kaufman *et al.*, *Proc. Nat'l. Acad.*

Sci. USA 83 3136-3140 (1986)). The examples below show the use of the *neo* (aminoglycoside phosphotransferase) gene, although the *hygro*, *his*, *dhfr*, *gpt*, *gs* or *ada* genes could also be used, as could any other suitable drug resistance gene. It should be noted that the use of the term "gene" in this context does not imply that natural genomic DNA has to be used, although that may be preferred. cDNAs may be at least as suitable, as may "minigenes" which contain some, but not all, of the introns which may naturally be present in the gene.

In another important embodiment, a DNA sequence whose expression gives rise to cell death (for example a toxin gene) is selectively expressed in differentiated cells. For this embodiment, the use of a promoter specific or substantially specific for differentiated cells is important, and the embryo cells can be made transgenic for, or otherwise be able to express, a construct comprising such a promoter operatively coupled to the lethal DNA sequence (for example that encoding the toxin).

One suitable promoter is that of the transforming growth factor β-2 gene, which is activated on differentiation of ES cells independent of the cell type formed (Mummery *et al.*, *Dev. Biol.* **137** 161-170 (1990)).

Genes encoding various toxins may be placed under the control of a promoter active in differentiated cells. For example, the Diphtheria toxin subunit-A gene (Maxwell *et al.*, *Mol. Cell. Biol.* 7 1576-1589 (1987)) or the Ricin toxin subunit-A gene (Landel *et al.*, *Genes and Dev.* **2** 1168-1178 (1988)) can be placed under the control of a promoter expressed only in differentiated cells. Again,

either the natural gene or a non-natural sequence encoding the toxin may be used.

Embryos which can express either (a) a selectable marker gene under the control of a promoter expressed specifically in undifferentiated ES cells and/or (b) a toxin gene under the control of a promoter expressed specifically in differentiated cells can be generated using standard methods. Such embryos may be transgenic, in that a heterologous DNA construct may be stably integrated in the embryonic genome, or they may simply contain non-integrated expressible DNA. ES cells may then be derived from these transgenic, injected or transfected embryos by culture under selective conditions.

Embryos suitable for use in the above method are also within the scope of the invention, according to a second aspect of which there is provided an embryo comprising expressible DNA comprising (a) a drug resistance gene (or other DNA sequence conferring drug resistance) under the control of a promoter expressed specifically in undifferentiated ES cells and/or (b) a DNA sequence whose expression gives rise to cell death (such as a toxin gene or other DNA sequence encoding a toxin) under the control of a promoter expressed specifically in differentiated cells. The embryo may be transgenic, in which case a transgene construct comprising the expressible DNA specified under (a) or (b) above may be integrated into the embryonic genome.

A transgenic embryo as in (a) above may be prepared either *de novo* or may be derived from a transgenic parent. A transgenic embryo as in (b) above would be

prepared *de novo*, in the absence of special measures, as expression of a toxin construct in differentiated cells would be lethal to a whole animal. Transgenic animals therefore also form a part of the present invention, according to a third aspect of which there is provided a transgenic, generally non-human, animal having integrated in its genome a transgene construct comprising a drug resistance gene (or other DNA sequence conferring drug resistance) under the control of a promoter expressed specifically in undifferentiated ES cells.

Embryos containing selectable DNA constructs as in (a) above may be obtained either by mating adult animals, at least one of which is transgenic, followed by embryo removal from the reproductive tract or by *de novo* transgenesis performed on the embryo. Transgenesis may be achieved, for example, by DNA microinjection or transfection. Microinjection methods involve DNA microinjection into zygotes or early cleavage stage embryos. Methods for transgenic mice are described in detail by Hogan *et al.*, "Manipulating the Mouse Embryo: A Laboratory Manual", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1986). DNA microinjection using essentially the same techniques has been successfully applied to other species, including farm animals, albeit with reduced efficiency (reviewed by Wall *et al.*, *Theriogenology* **38** 337-357 (1992) and Wilmut *et al.*, *Reprod. Fert. Suppl.* **43** 265-275 (1991)). Transgenic animals may be identified by DNA analysis of tissue biopsies, and appropriate matings carried out to produce transgenic embryos.

It is not envisaged that such transgene constructs will necessarily be the only transgene constructs integrated

into the animal's genome, although in many cases they will be the only ones.

Not all embryos in accordance with the second aspect of the invention will be transgenic. An alternative is for an expression construct simply to be introduced into the embryo cell(s) by any suitable means, such as microinjection (see, for example, Burdon *et al.*, *Mol. Reprod. and Dev.* **33** 436-442 (1992)). Although such a construct may not survive for long in the absence of replication sequences (and in the absence of integration) it may persist for long enough for the method of the first aspect of the invention to be practised on the embryo. In any event, the relatively short duration of a non-integrating, non-replicating construct may actually be an advantage, as no particular steps are needed to remove the construct if its continued presence is either not desired, or where its removal is obligatory. The latter case would apply to ES cells containing a toxin gene under the control of a promoter causing specific expression in differentiated cells. Such a construct is normally to be removed before ES cells can participate in embryo development.

A third possibility is that the expression construct may be present in the embryonic cells coupled to sequences that give rise to episomal replication. Episomal vectors of this type have been derived from bovine papilloma virus (Mathias *et al.*, *EMBO J.* **2** 1487-1492 (1983)) and adenovirus (Quantin *et al.*, *Proc. Nat'l. Acad. Sci.* **89** 2581-2584 (1992)). The advantage of using an episomal vector is that most embryos and their cultured derivatives will contain the selective cassette for an extended period after microinjection.

Selection strategies for ES cell isolation depend upon expression of ES-specific salvage transgenes which preserve the ES lineage under generally lethal selection conditions or differentiated cell-specific expression of toxin transgenes. Integration and maintenance of the transgene after ES isolation is probably unimportant and may be undesirable. A short term transfection system such as lipofection (Felgner *et al.*, *Nature* **337** 387-388 (1989)) is therefore suited to this approach in large animals -- particularly where transient infection can be effected under near-physiological conditions (Brunette *et al.*, *Nucl. Acids Res.* **20** 1151 (1992)). Lipofection involves the spontaneous association of DNA with a liposome containing a cationic lipid, which then fuses with the cell membrane, leading to internalisation of DNA. Suitable liposome preparations are commercially available.

There are a number of advantages of lipofection as a means of transgenesis in the context of the present invention. First, as a procedure, it takes far less effort and time than microinjection; therefore, large numbers of embryos can be treated. Secondly, lipofection is a relatively benign treatment which can be applied repeatedly without toxicity. Repeated lipofection will therefore help ensure the presence of DNA in a high proportion of explanted embryos. Thirdly, selectable DNA will be present mainly as non-integrated, non-replicating molecules; it should therefore be easy to isolate non-transgenic ES cells once selection is removed.

DNA constructs useful in the preparation of embryos as described above are also within the scope of the invention, according to a fourth aspect of which there is

provided a DNA construct comprising (a) a drug resistance gene (or other DNA sequence conferring drug resistance) under the control of a promoter expressed specifically in undifferentiated ES cells and/or (b) a DNA sequence whose expression gives rise to cell death (such as a toxin gene or other DNA sequence encoding a toxin) under the control of a promoter expressed specifically in differentiated cells.

Once an ES cell line has been established the selectable marker or toxin gene construct can, if necessary or desirable, be removed. In the case of non-integrating, non-replicating DNA the removal is, in effect, automatic. For transgenic embryos and embryos in which the expression construct is episomally replicating, more deliberate steps have to be taken. The use of site specific recombination systems to generate precisely defined deletions in cultured mammalian cells has recently been demonstrated. Gu *et al.* (*Cell* **73** 1155-1164 (1993)) describe how a deletion in the immunoglobulin switch region in mouse ES cells was generated between two copies of the bacteriophage P1 *lox*P site by transient expression of the Cre site-specific recombinase, leaving a single *lox*P site. Similarly, yeast FLP recombinase has been used to precisely delete a selectable marker defined by recombinase target sites in mouse erythroleukemia cells (Fiering *et al.*, *Proc. Nat'l. Acad. Sci. USA* **90** 8469-8473 (1993)). The Cre *lox* system is exemplified below, but other site-specific recombinase systems could be used.

A construct used in the Cre *lox* system will usually have the following three functional elements:

1. The expression cassette;

2. A negative selectable marker (e.g. Herpes simplex virus thymidine kinase (TK) gene) expressed under the control of a ubiquitously expressed promoter (e.g. phosphoglycerate kinase (Soriano *et al.*, *Cell* **64** 693-702 (1991)); and

3. Two copies of the bacteriophage P1 site specific recombination site *lox*P (Baubonis *et al.*, *Nuc. Acids. Res.* **21** 2025-2029 (1993)) located at either end of the DNA fragment.

This construct can be eliminated from established ES cell lines containing it by means of site specific recombination between the two *lox*P sites mediated by Cre recombinase protein which can be introduced into the cells by lipofection (Baubonis *et al.*, *Nuc. Acids Res.* **21** 2025-2029 (1993)). Cells which have deleted DNA between the two *lox*P sites are selected for loss of the TK gene (or other negative selectable marker) by growth in medium containing the appropriate drug (ganciclovir in the case of TK).

Embryonic stem cells isolated by the selection procedure can be tested for totipotency by assessing their capacity to form adult tissues, most importantly germ cells. Totipotent embryonic stem cells can be used as a means of manipulating an animal's genome either by simple introduction of a transgene or by the more extensive modifications possible using gene targeting technology.

Preferred features of each aspect of the invention are as for each other aspect *mutatis mutandis*.

The invention will now be illustrated by the following examples. The examples refer to the drawings, in which:

FIGURES 1A to 1F show representative examples of embryoid bodies obtained as described in Example 2; and

FIGURE 2 shows the structure of the OctneoTL construct.

EXAMPLE 1 Generation of Octneo DNA Construct

A 1.94 kb fragment containing the 5'-flank and promoter region of the Oct-3/4 gene was amplified from mouse genomic DNA by the polymerase chain reaction. The sequence of primers used corresponded to the Oct-3/4 published sequence (Okazawa *et al.*, *EMBO J.* **10** 2997-3005 (1991)) with additional restriction enzyme cleavage sites to facilitate cloning and construction. The Oct-3/4 fragment was used to generate a construct, designated Octneo, designed to confer resistance to the drug G418 on undifferentiated ES cells.

Octneo was constructed from three DNA fragments:

1. A 1.94 kb Oct-3/4 5' flanking fragment (1940bp to 34bp upstream of the translation initiation site) with 5' *Sal*I and 3' *Hin*dIII sites derived from the PCR primers;

2. A 1.7 kb *Hin*dIII/*Eco*RI fragment containing a modified aminoglycoside phosphotransferase (*neo*) gene and human growth hormone polyadenylation signal (Selfridge *et al.*, *Som. Cell and Mol. Gen.* **18** 325-336 (1992)); and

3. pUC18 cut with *Eco*RI and *Sal*I.

EXAMPLE 2 ES Cell Specific Expression of Octneo Gene

The tissue specificity of Oct 3/4-directed *neo* expression was investigated in two experiments:

2.1 Octneo expression in ES cells and fibroblasts.

Octneo DNA was transfected into NIH 3T3 differentiated fibroblasts and HM-1 undifferentiated ES cells (Magin *et al.*, *Nuc. Acids. Res.* **14** 3795 (1992)). A control construct, designated MTneo, in which *neo* expression is directed by the metallothionein promoter, was transfected in parallel. Colony numbers observed after G418 selection are shown in Table 1:

Table 1.

Cell line	Octneo	MTneo
NIH3T3	0	31
HM-1	11	9

This indicates that the Oct 3/4 promoter is active in ES cells and inactive in fibroblasts.

2.2 Effects of Octneo expression on *in vitro* differentiation of ES cells under G418 selection

Octneo and MTneo constructs were transfected by electroporation into HM-1 ES cells and G418-resistant clones isolated. A suspension culture method of inducing differentiation of ES cells was then used. The method was very similar to that described by Robertson, E.J. "Embryo-Derived Stem Cells" in: "Teratocarcinomas and Embryonic Stem Cells, A Practical Approach", Ed. E.J. Robertson, Pub. IRL Press Ltd., Oxford, 1987. Such differences from the Robertson method that there were are detailed in the following description.

Differentiation of transfected clones under selective conditions was investigated as now described.

Day 0: Two Octneo clones (Octneo 2,4) and one MTneo clone (MTneo 6) were grown as undifferentiated ES cell monolayers on gelatinised tissue culture plastic in ES culture medium as described by Selfridge *et al.* (*Som. Cell and Mol. Gen.* **18** 325-336 (1992)). G418 was also added at 0.2mg/ml. This concentration of G418 is double that necessary to kill untransfected HM-1 cells.

Day 1: ES cell monolayers passaged onto non-gelatinised plastic in ES culture medium to induce aggregation of cells.

Day 2: ES cells passaged as multicellular aggregates into suspension culture in bacterial petri dishes. The culture medium used was ES medium with reduced serum (2.5% foetal, 2.5% newborn serum) and LIF omitted. After a few days under these conditions aggregates of ES cells tend to form embryoid bodies composed of an internal mass of undifferentiated cells and an outer layer of endoderm.

Cells from each clone were divided into four groups each with different G418 concentrations in the culture medium: 0mg/ml, 0.5mg/ml, 1.0mg/ml, 1.5mg/ml.

Day 8: Embryoid bodies were induced to attach to a substrate by transfer to gelatinised tissue culture dishes. This was to allow assessment of the extent of differentiation which had occurred in suspension culture. However, reattachment itself provides a stimulus to differentiate and so G418 selection was maintained for a further week to allow newly differentiated cells to be

killed. During this time growth was in ES culture medium including LIF. The presence of LIF does not affect the viability of differentiated cells.

Day 15: Attached embryoid bodies were fixed with methanol/acetic acid and stained with crystal violet. Differentiation was scored by the presence of a halo of differentiated cells, predominantly endoderm, spreading out onto the substrate.

Results are summarised in Table 2 and representative examples of embryoid bodies are shown in Figures 1A to 1F.

Table 2. Differentiation of embryoid bodies under selective conditions.

	G418 concn.			
Clone	0mg/ml	0.5mg/ml	1mg/ml	1.5mg/ml
MTneo 6	+	+	+	+
Octneo 2	+	-	-	-
Octneo 4	+	-	-	-

+ Extensive differentiation.
- Little or no differentiation.

These results show that differentiated ES derivatives of Octneo transfected clones are killed at G418 concentrations of 0.5mg/ml and 1mg/ml, while differentiated derivatives of the MTneo control survive. At higher levels of G418 a more general toxicity in both differentiated and undifferentiated cell types was observed.

EXAMPLE 3 ES cell derivation from Octneo transgenic murine embryos

The effect of drug selection on the efficiency of ES cell derivation was investigated. Octneo DNA was micro-injected into mouse zygotes and 10 transgenic mouse lines (C57B1 / CBA hybrid) were generated by standard techniques (Hogan *et al.*, *loc cit*).

Transgenic male offspring of founder animals from each transgenic line were mated naturally with strain 129SV females. Blastocysts were collected and explanted intact onto feeder layers using the procedure described by Robertson (*loc cit*). Feeder layers were mitomycin -C inactivated STO-neo cells, which are an established fibroblast line transfected with a metallothionein-neo construct conferring resistance to G418. Each flush (ie the contents of a single uterus) was divided in a ratio of 1:2 into non-selective and selective treatments. Selection was carried out with 100 μg/ml G418 included in the culture medium. This concentration had been previously established as the minimum necessary to kill non-transgenic embryos within 10 days. Primary embryo explants were cultured as pools of 2-5 embryos in single wells of 48 well plates. The culture medium consisted of BHK21 Glasgow MEM, supplemented with 10% newborn and 10% fetal serum and murine leukaemia inhibitory factor. In all other respects ES cell isolation proceeded as described by Robertson (*loc cit*).

Embryos from 5 of the transgenic mouse lines all died under selective conditions as shown below:

Lines	Embryos	Explants (+G418)	ES lines (+G418)	Explants (-G418)	ES lines (-G418)
72-92	19	13	0	6	0
72-28	13	9	0	4	0
71-12	23	16	0	7	0
71-10	0	0	0	0	0
71-19	30	20	0	10	0
Total	**85**	**58**	**0**	**27**	**0**

However, embryos from the other 5 lines gave rise to G418 resistant ES cell lines. These data are shown below:

Line	Embryos	Explants (+G418)	ES lines (+G418)	Explants (-G418)	ES lines (-G418)
72-9	25	17	4	8	0
72-49	17	10	1	7	2
71-2	12	10	2	2	0
71-11	43	26	2	17	0
72-10	20	11	1	9	2
Total	**117**	**74**	**10**	**43**	**4**

As the stud males used were hemizygous for the Octneo transgene, half of the explanted embryos did not contain the Octneo transgene and so could not survive G418 selection. When comparing the rate of ES cell derivation under selective and non selective conditions, it is therefore necessary to double the efficiency of ES cell derivation under selection to take account of this mortality.

Thus:

ES derivation efficiency of in the absence of G418: **9%**

Non adjusted efficiency in the presence of G418: 13.5%,

True efficiency, accounting for death of non-transgenic embryos: **27%**

Statistical significance (by unrelated t test), $p < 0.05$.

These results indicate that G418 selection of Octneo transgenic embryos significantly improves the efficiency of ES cell line derivation.

EXAMPLE 4 Derivation of ES Cells which do not Contain Octneo DNA

In many cases it is desirable that ES cells do not contain experimentally introduced DNA sequences. A modified drug selection construct can be used to establish an ES cell line and may then be removed from the host genome. This example uses the bacteriophage P1 site-specific recombinase Cre and its target site *lox*P. The construct used to exemplify this is designated OctneoTL and is composed, as is shown in Figure 2, of three functional elements:

1. The drug selection cassette (Octneo);

2. A negative selectable marker (e.g. Herpes simplex virus thymidine kinase (TK) gene) expressed under the control of a ubiquitously expressed promoter (e.g. phosphoglycerate kinase (Soriano *et al.*, *Cell* **64** 693-702 (1991)); and

3. Two copies of the bacteriophage P1 site specific recombination site *lox*P (Baubonis *et al.*, *Nuc. Acids. Res.* **21** 2025-2029 (1993)) located at either end of the DNA fragment.

A strain of Octneo TL transgenic animals are generated as in Example 3 and ES cells are derived from transgenic embryos by culture with G418 selection. Established ES cell lines can be rendered non-transgenic by means of site specific recombination between the two *lox*P sites

mediated by Cre recombinase protein which can be introduced into the cells by lipofection (Baubonis *et al.*, *Nuc. Acids Res.* **21** 2025-2029 (1993)). Cells which have deleted DNA between the two *lox*P sites are selected for loss of the TK gene by growth in medium containing the drug ganciclovir.

EXAMPLE 5 Derivation of ES Cells from Large Animal Species

The generation of a transgenic strain for the purpose of ES cell derivation is impractical in domestic livestock such as cattle because of the lengthy generation time. In this case, the selectable construct is microinjected or transfected into embryos which are then placed directly into culture and ES cells derived.

The selective cassette can be introduced into livestock embryos as a DNA fragment as described in Examples 3 or 4, or alternatively in a vector designed to replicate independently of the host genome. Episomal vectors of this type have been derived from bovine papilloma virus (Mathias *et al.*, *EMBO J.* **2** 1487-1492 (1983)) and adenovirus (Quantin *et al.*, *Proc. Nat'l. Acad. Sci.* **89** 2581-2584 (1992)). The advantage of using an episomal vector is that most embryos and their cultured derivatives will contain the selective cassette for an extended period after microinjection. In contrast, only 10-15% of embryos microinjected with a conventional DNA fragment will contain stably integrated DNA. Unintegrated DNA persists in other embryos for approximately one week after injection.

Removal of the episome from established lines can be achieved by ganciclovir selection against the TK marker gene.

CLAIMS

1. A method for selecting embryonic stem (ES) cells from an embryo in culture, the method comprising selectively killing differentiated cells of the embryo.

2. A method as claimed in claim 1, wherein the differentiated cells are killed by means of a drug selection regime.

3. A method as claimed in claim 2, wherein embryo cells contain a construct comprising a promoter specific or substantially specific for undifferentiated cells operatively coupled to a DNA sequence encoding a drug resistance marker.

4. A method as claimed in claim 3, wherein the promoter is the Oct-3/4 promoter.

5. A method as claimed in claim 3, wherein the promoter is the FGF-4 promoter.

6. A method as claimed in claim 3, 4 or 5, wherein the drug resistance marker is encodable by the *neo*, *hygro* or *his*, *dhfr*, *gpt*, *gs* or *ada* gene.

7. A method as claimed in claim 1, wherein a DNA sequence whose expression gives rise to cell death is selectively expressed in differentiated cells.

8. A method as claimed in claim 7, wherein embryo cells contain a construct comprising a promoter specific or substantially specific for differentiated cells operatively coupled to a DNA sequence whose expression gives rise to cell death.

9. A method as claimed in claim 8, wherein the DNA sequence whose expression gives rise to cell death encodes a toxin.

10. A method as claimed in claim 9, wherein the toxin is diphtheria toxin or ricin toxin.

11. A method as claimed in any one of claims 3 to 6 and 8 to 10, in which the embryo is transgenic for the construct.

12. A method as claimed in any one of claims 3 to 6 and 8 to 10, in which the construct is capable of episomal replication within the embryo.

13. A method as claimed in any one of claims 3 to 6 and 8 to 10, in which the construct is present within the embryo in non-integrated and non-replicating form.

14. An animal embryo comprising expressible DNA comprising (a) a DNA sequence conferring drug resistance under the control of a promoter expressed specifically in undifferentiated ES cells and/or (b) a DNA sequence whose expression gives rise to cell death under the control of a promoter expressed specifically in differentiated cells.

15. An embryo as claimed in claim 14, in which the embryo is transgenic for the expressible DNA.

16. An embryo as claimed in claim 14, in which expressible DNA is capable of episomal replication within the embryo.

17. An embryo as claimed in claim 16, wherein the expressible DNA is present on a bovine papilloma virus expression vector.

18. An embryo as claimed in claim 16, wherein the expressible DNA is present on an adenovirus expression vector.

19. An embryo as claimed in claim 14, in which the expressible DNA is present within the embryo in non-integrated and non-replicating form.

20. A transgenic animal having integrated in its genome a transgene construct comprising a DNA sequence conferring drug resistance under the control of a promoter expressed specifically in undifferentiated ES cells.

21. A DNA construct comprising (a) DNA sequence conferring drug resistance under the control of a promoter expressed specifically in undifferentiated ES cells and/or (b) a DNA sequence whose expression gives rise to cell death under the control of a promoter expressed specifically in differentiated cells.

22. A method as claimed in any one of claims 3 to 6 and 8 to 10, in which the construct is subsequently removed from embryo cells.

23. A method as claimed in claim 22, in which the construct is removed by a use of a site-specific recombination system.

24. A method as claimed in claim 23, wherein the site-specific recombination system is the Cre*lox* system or the FLP system.

25. A method as claimed in claim 22, 23 or 24, wherein removal of the construct is selected for by means of a negatively selectable marker, which may be under the control of a constitutive promoter.

26. A method as claimed in claim 25, wherein the negatively selectable marker is the Herpes Simplex Virus thymidine kinase (TK) gene.

27. A method as claimed in claim 25 or 26, wherein the constitutive promoter is the phosphoglycerate kinase promoter.

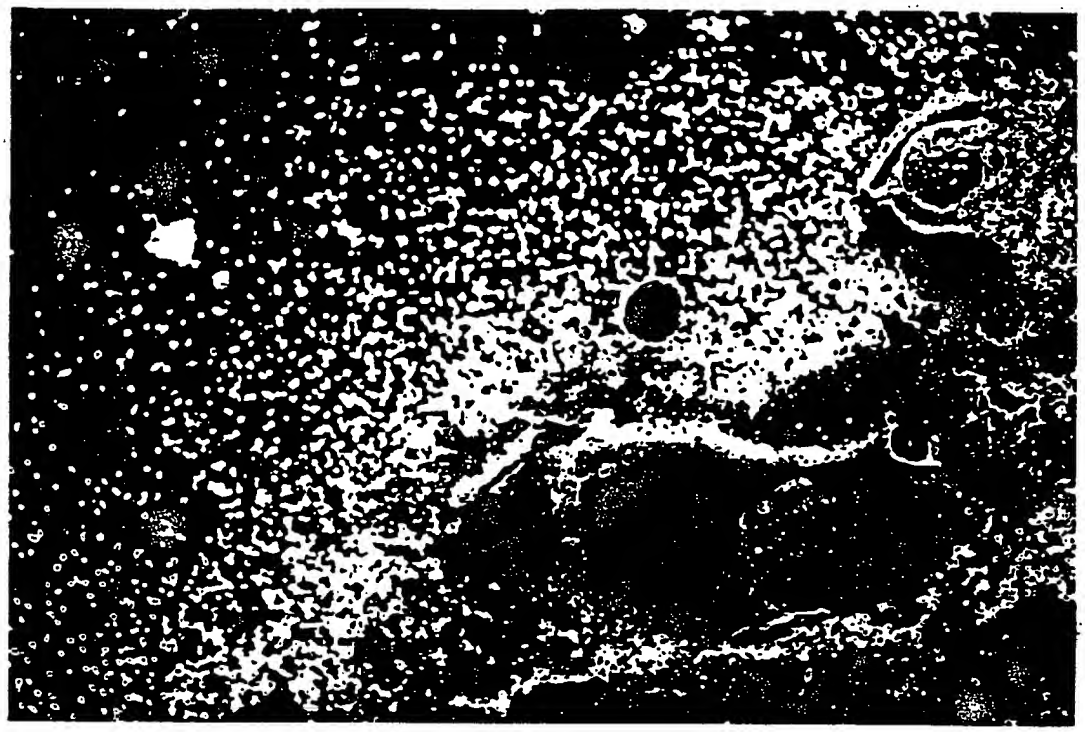

FIGURE 1A

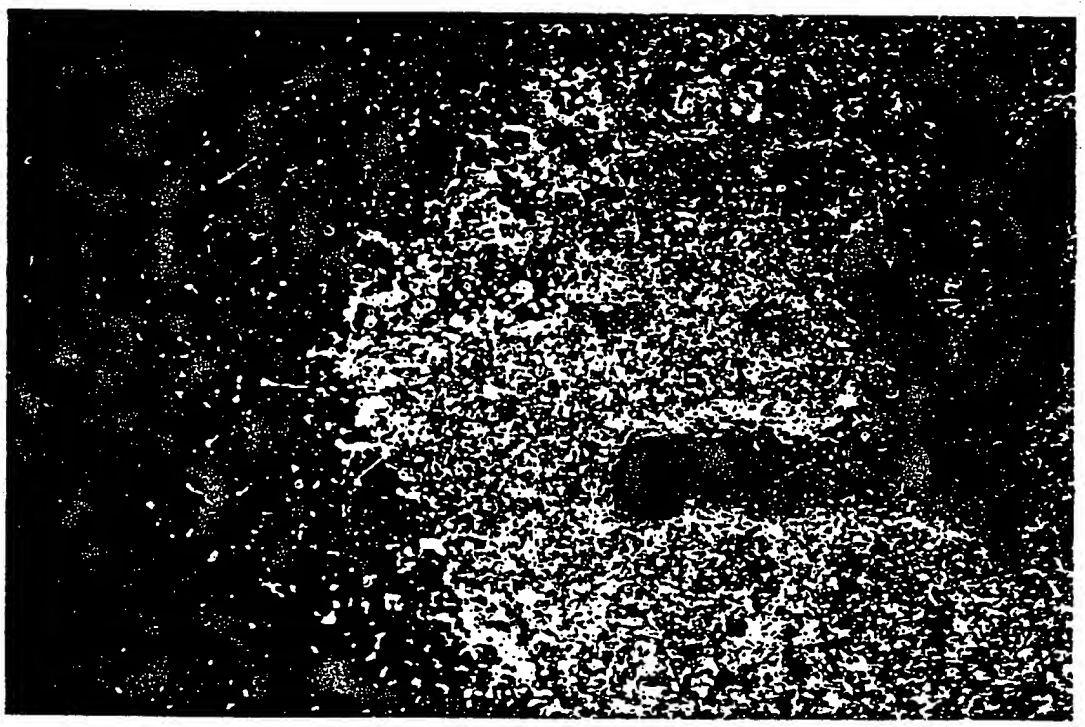

FIGURE 1B

FIGURE 1C

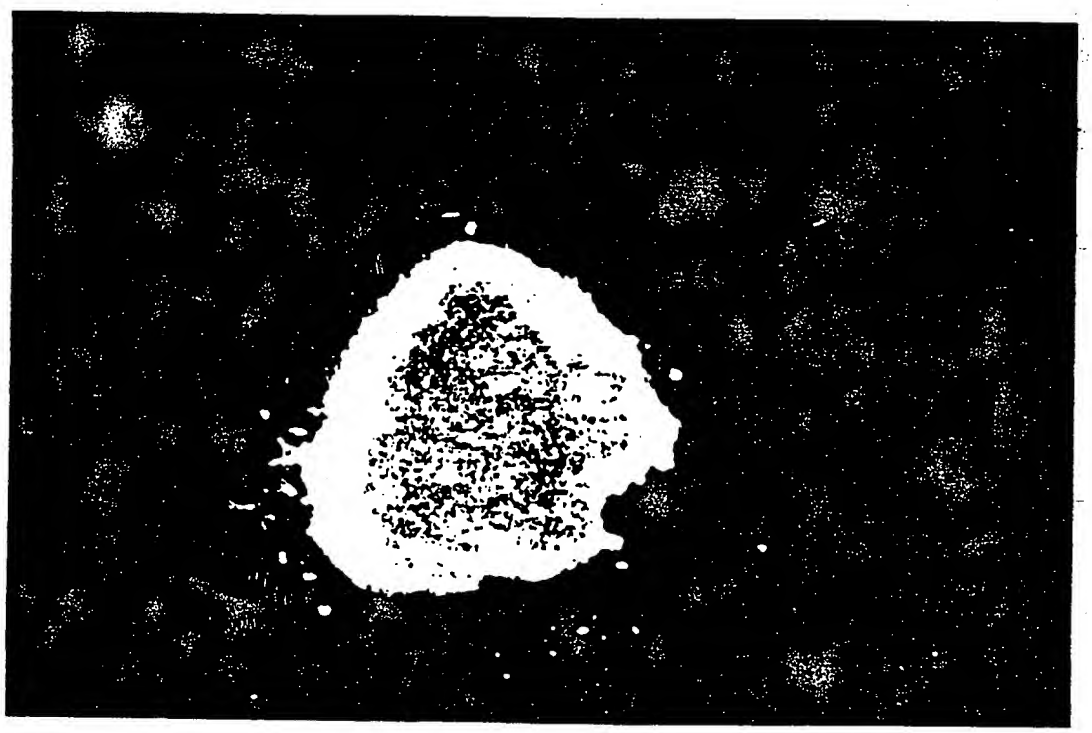

FIGURE 1D

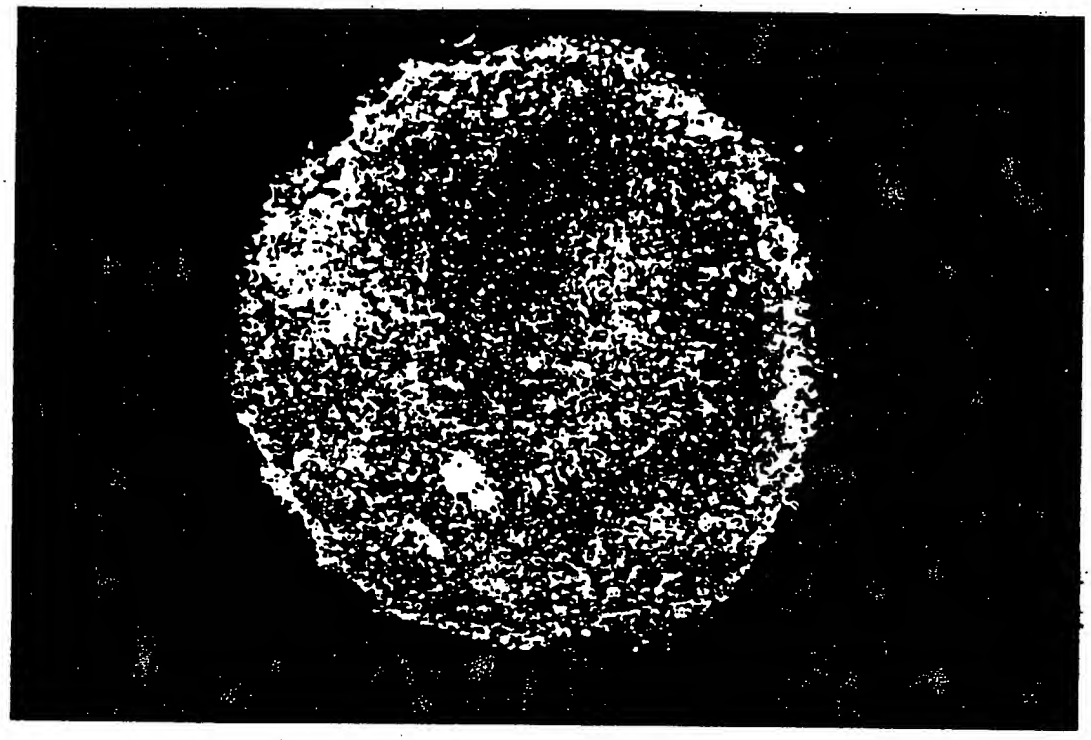

FIGURE 1E

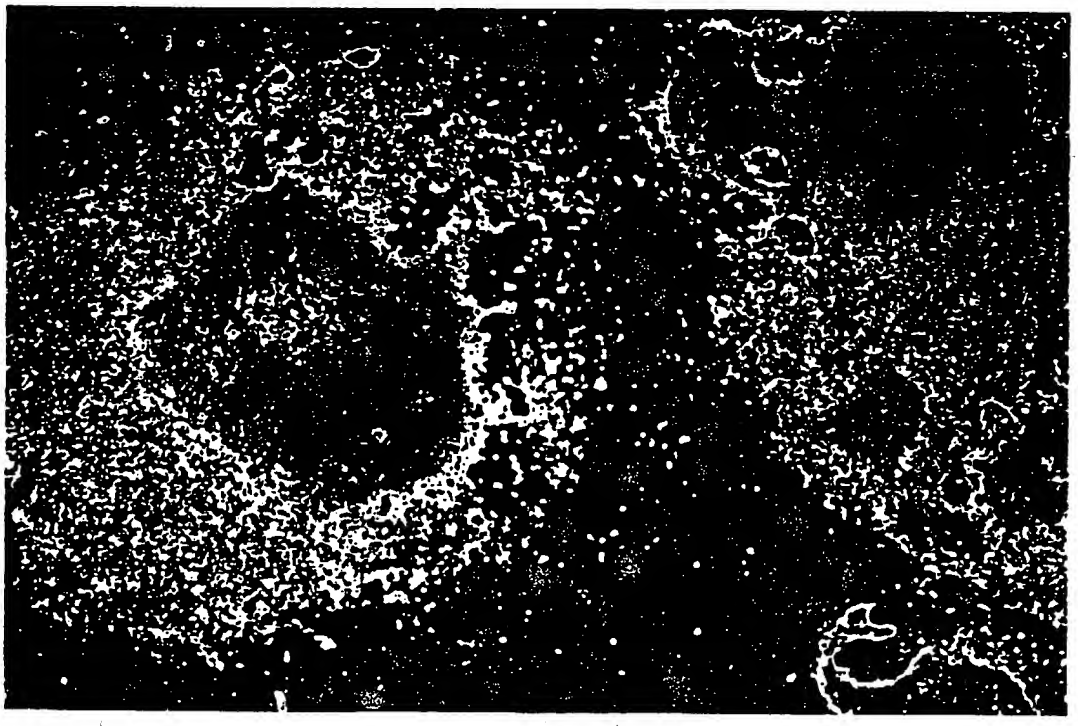

FIGURE 1F

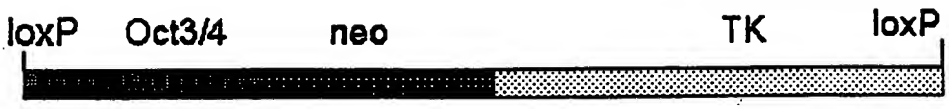

FIGURE 2

INTERNATIONAL SEARCH REPORT

In tional Application No
PCT/GB 95/00140

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/10 A01K67/027 C12N15/85

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO,A,94 24274 (UNIVERSITY OF EDINBURGH) 27 October 1994 see the whole document ---	1-27
A	DEV. BIOL., vol. 154, no. 1, 1992 pages 45-54, Y. MA ET AL. 'Transcriptional regulation of the murine k.FGF gene in embryonic cell lines.' cited in the application see the whole document --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
14 June 1995	29.06.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Skelly, J

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EMBO J., vol. 10, no. 10, 1991 pages 2997-3005, H. OKAZAWA ET AL. 'The oct3 gene a gene for an embryonic transcription factor is controlled by a retinoic acid repressible enhancer' cited in the application see the whole document	1
A	WO,A,90 03432 (ANIMAL BIOTECHNOLOGY CAMBRIDGE LTD) 5 April 1990	
A	WO,A,90 01541 (AMRAD CORPORATION LTD) 22 February 1990	

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Ir. .tional Application No
PCT/GB 95/00140

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		AU-B-	6542794	08-11-94
		WO-A-	9424301	27-10-94
WO-A-9003432	05-04-90	AU-B-	650616	30-06-94
		AU-A-	4321789	18-04-90
		EP-A-	0435928	10-07-91
		JP-T-	4500909	20-02-92
WO-A-9001541	22-02-90	AU-B-	623922	28-05-92
		AU-A-	4059089	05-03-90
		EP-A-	0380646	08-08-90
		JP-T-	3503241	25-07-91
		US-A-	5166065	24-11-92